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Supplemental Information

Glutamine Triggers Acetylation-Dependent Degradation of Glutamine Synthetase via the Thalidomide Receptor Cereblon

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Supplemental Figures and Legends

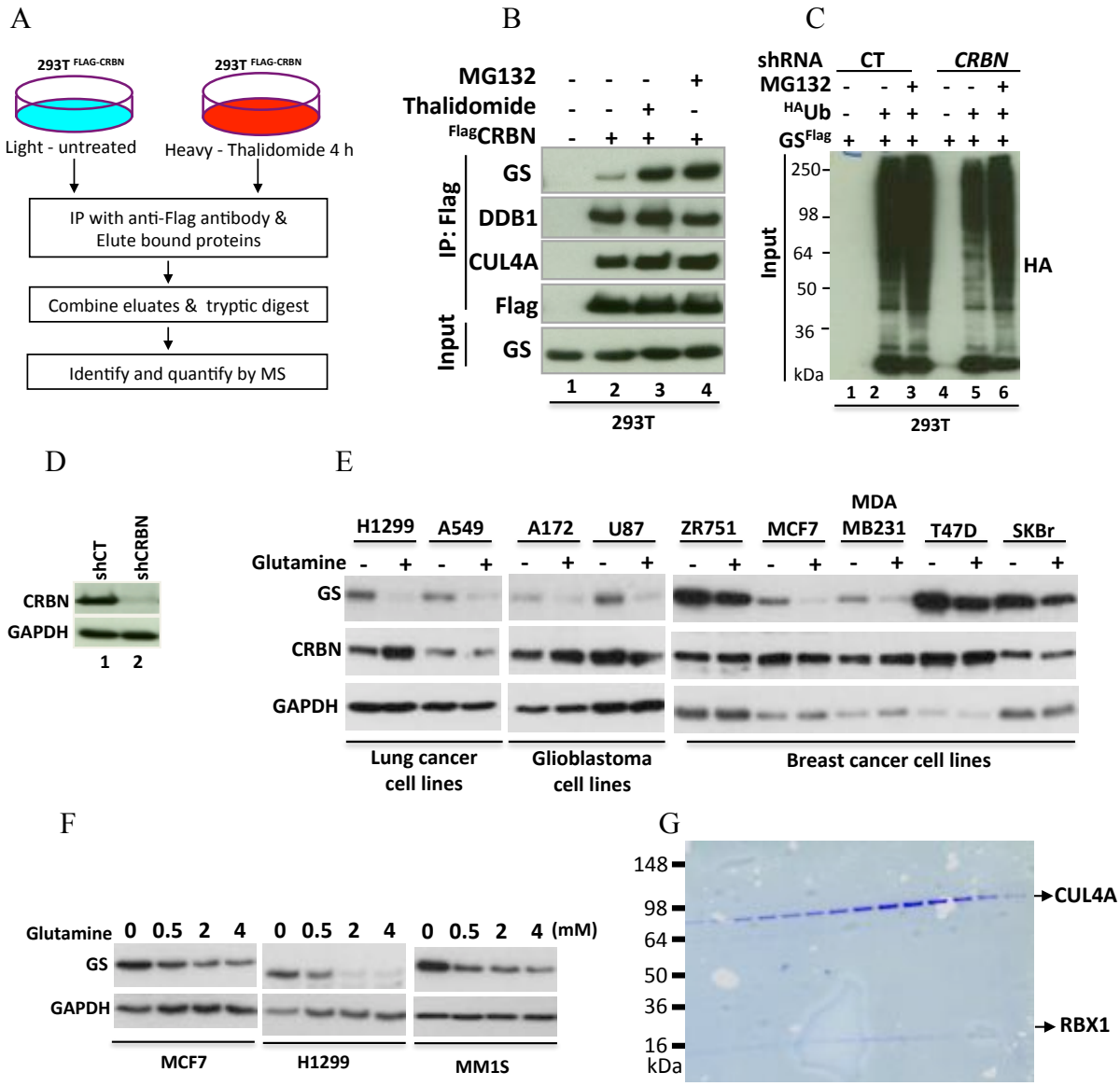


Figure S1. Identification of GS as a CRBN-interacting protein, Related to Figure 1 and Figure 2.

(A) Schematic diagram of the SILAC screen for thalidomide-modulated CRBN-interacting proteins. We generated a 293T cell line that stably expressed CRBN with a Flag tag appended to its amino terminus (^{Flag}CRBN). ^{Flag}CRBN cells were grown in medium formulated with isotopically light lysine and arginine (light medium) or in medium formulated with isotopically heavy lysine and arginine (heavy medium). Cells growing in heavy medium were treated with 50 μM thalidomide for 4 h, whereas cells growing in light medium were treated with DMSO.

^{Flag}CRBN immunoprecipitates were prepared in parallel, mixed, and analyzed by quantitative mass spectrometry.

(B) Validation of mass spectrometry. 293T cells stably expressing ^{Flag}CRBN (lanes 2–4) were treated with 50 μM thalidomide (lane 3) or 10 μM MG132 (lane 4) for 4 h prior to lysis and immunoprecipitation (IP) with anti-Flag resin. Immunoprecipitates and input samples were fractionated by SDS-PAGE and immunoblotted (IB) with antibodies to detect ^{Flag}CRBN and endogenous GS, DDB1, and CUL4A.

(C) Western blot analysis showing input protein levels for *in vivo* ubiquitylation assay corresponding to Figure 1D.

(D) Confirmation of CRBN depletion. 293T cells were infected with control lentivirus (non-target shRNA), or lentivirus expressing *CRBN* shRNA. After 3 weeks of puromycin selection, cell extracts were analyzed by SDS-PAGE and immunoblotting with anti-CRBN antibody or anti-GAPDH antibody (control). This blot served as a control for Figure 1D.

(E) Glutamine mediates GS downregulation in multiple cancer cell lines. The indicated cell lines were starved of glutamine for 24 h, and then treated with glutamine (4 mM) for 10 h. Equal amounts of protein extracts were analyzed by immunoblotting with the indicated antibodies.

(F) Titration of glutamine. MCF7, H1299, and MM.1S cells were maintained in complete medium containing the indicated glutamine concentrations for 24 h. Protein lysates were analyzed by Western blotting with antibodies against GS and GAPDH.

(G) Purification of recombinant human CUL4A–RBX1. Fractions from the last purification step were analyzed by SDS-PAGE and staining with Coomassie Blue. This recombinant CUL4A-RBX1 was used in the *in vitro* ubiquitylation assay, shown in Figure 1E.

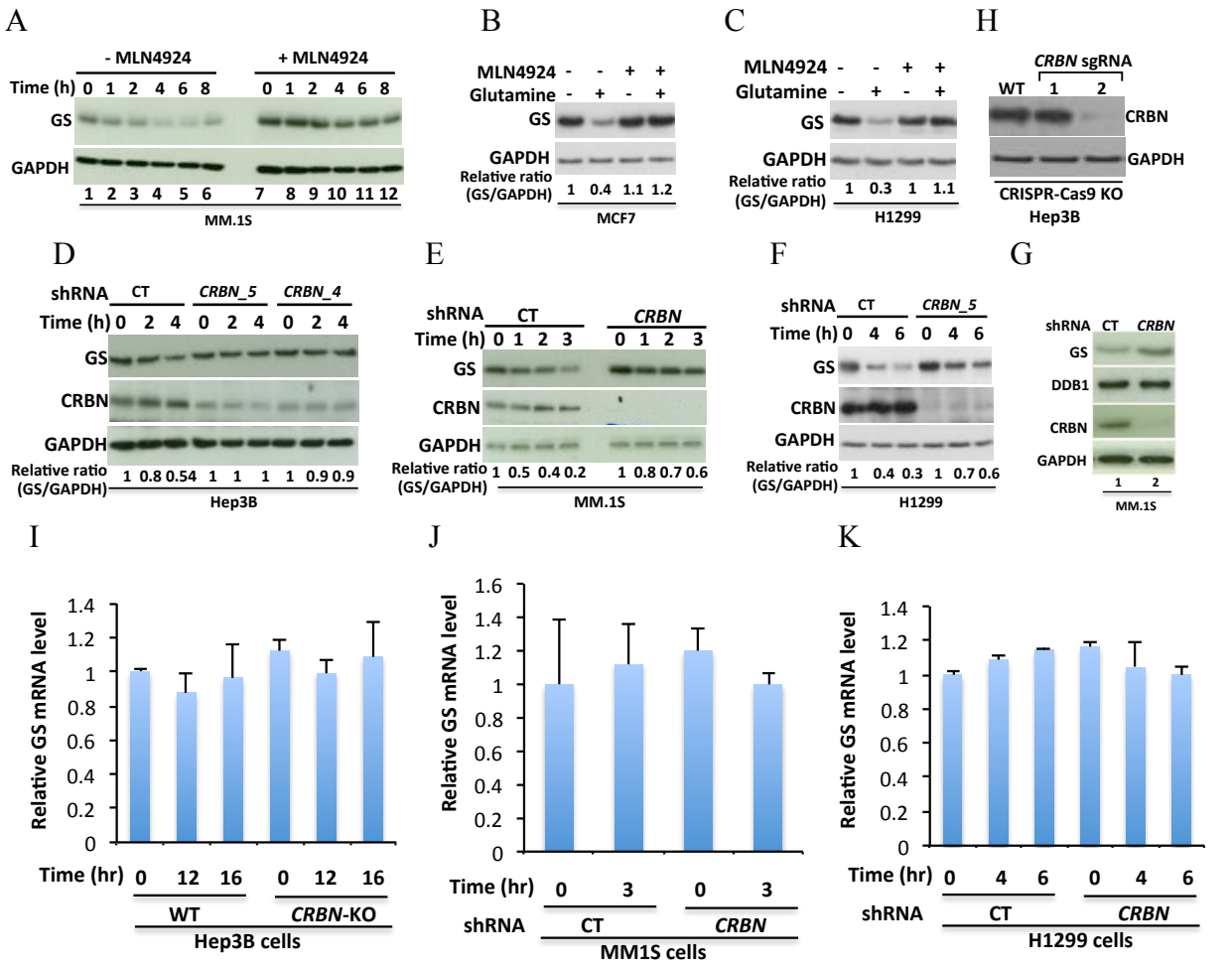


Figure S2. Glutamine-induced degradation of GS requires CRL4^{CRBN} activity, related to Figure 2.

(A-C) MLN4924 inhibits glutamine-induced GS degradation. MM.1S (A), MCF7 (B), and H1299 (C) cells were starved of glutamine for 24 h. Then the cells were treated with or without CRL inhibitor MLN 4924 (1μM) for 1 h, followed by addition of 4 mM glutamine for the indicated times (A) or for 6 h (B-C). Equal amounts of cell extracts were analyzed by SDS-PAGE and immunoblotting with antibodies against GS and GAPDH. In B and C, the GS:GAPDH ratio for each sample was calculated, normalized to untreated cells, and is indicated below the bottom panels.

(D-F) Depletion of CRBN impairs the glutamine-induced degradation of GS. Hep3B (D), MM.1S (E), and H1299 (F) cells stably expressing control shRNA or *CRBN* shRNA were starved of glutamine for 48, 24, or 24 h, respectively. Then the cells were treated with 4 mM glutamine for the indicated times. Cell extracts were analyzed by SDS-PAGE and immunoblotting with antibodies against GS, CRBN, and GAPDH (control). The GS:GAPDH ratio, normalized to that of untreated cells, is shown below each set of panels.

(G) GS accumulates in unperturbed cells depleted of CRBN. MM.1S cells were transduced with lentiviruses that expressed either control (CT) shRNA or *CRBN* shRNA. Forty-eight hours after transduction, cells were lysed, fractionated by SDS-PAGE, and immunoblotted with the indicated antibodies.

(H) Western blot showing the knockout (KO) efficiency by CRISPR gRNA *CRBN* in Hep3B cells. Protein lysates were analyzed by Western blotting with antibodies against CRBN and GAPDH. *CRBN*-KO Hep3B cells (*CRBN* sgRNA_2) were used in subsequent experiments, showed in Figure 2C and Figure S2I.

(I-K) The mRNA levels of *GS* remained unchanged in CRBN-depleted cells and glutamine-treated cells. The expression of *GS* in Hep3B (I), MM1S (J) and H1299 cells (K), starved of glutamine for 24 h followed by addition of 4 mM glutamine at the indicated times, was analyzed by quantitative RT-PCR. *GS* mRNA expression in each sample is normalized to *GAPDH* mRNA level. The relative abundance of *GS* mRNA levels in CRBN-depleted cells or in glutamine-treated cells was normalized to that of untreated control cells. The relative *GS* mRNA level is displayed as an average of triplicates of quantitative PCR in each sample, and error bars indicate ± SD.

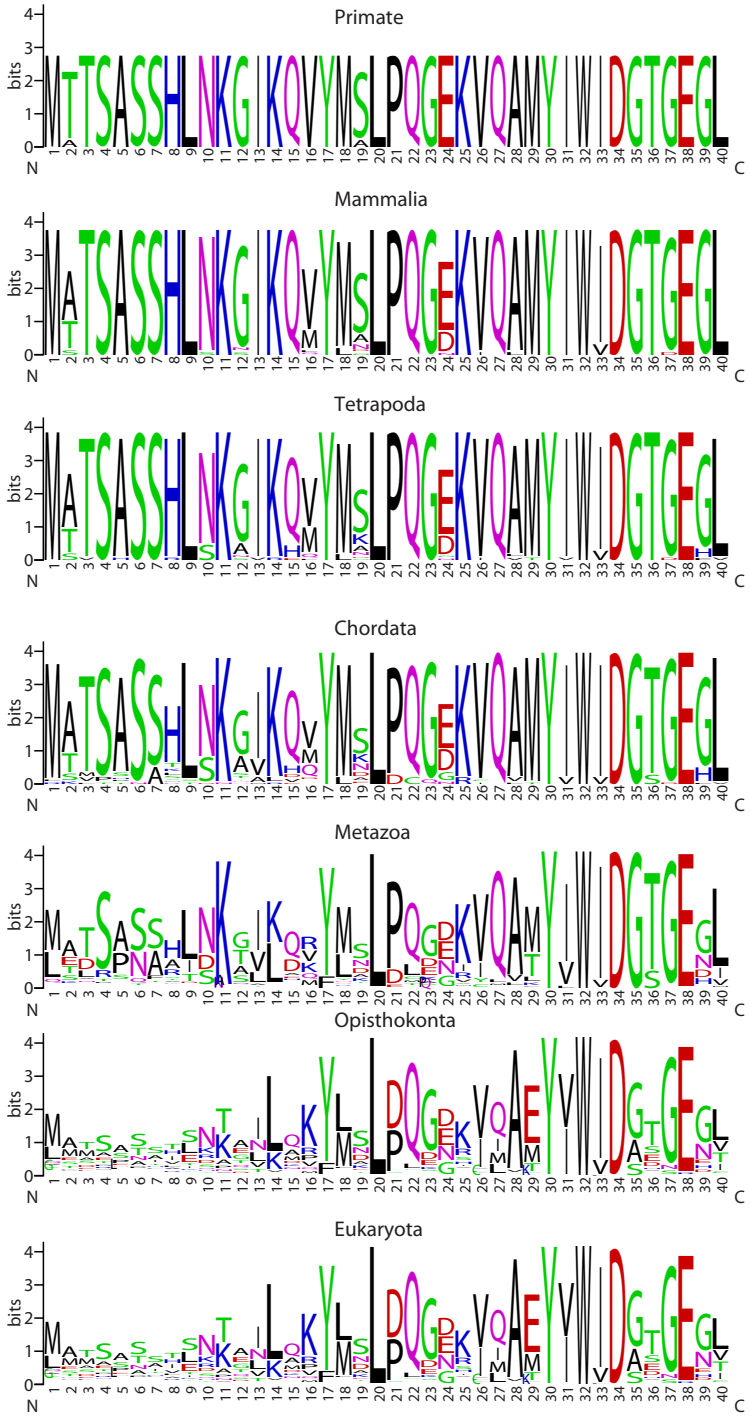


Figure S3. Conservation of the N-terminal extension of GS in major branches of the eukaryotic lineage, related to Figure 3. This is a graphic representation of amino acid sequence alignment, generated using WebLogo (http://weblogo.berkeley.edu/logo.cgi)

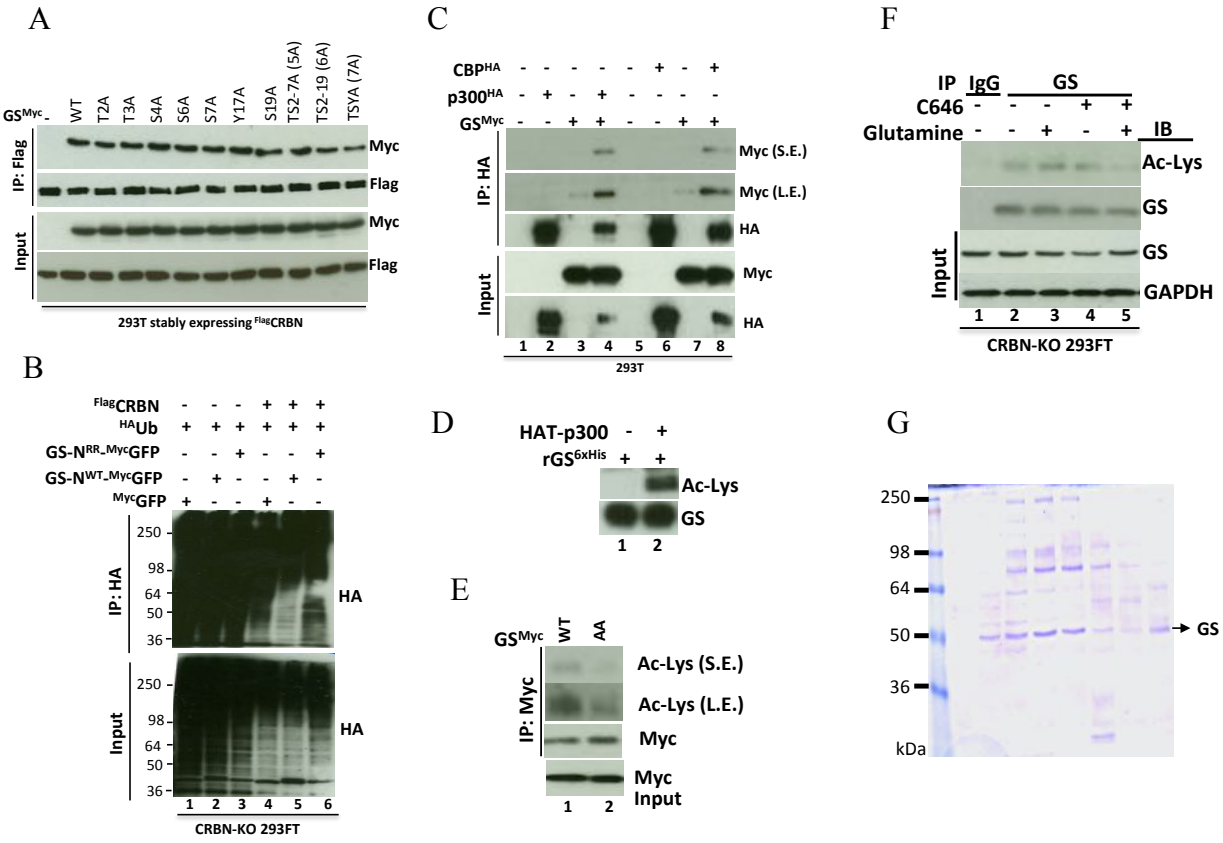


Figure S4. GS is acetylated *in vitro* and *in vivo* by p300, related to Figure 3, Figure 4 and Figure 5.

(A) Mutations of putative serine, threonine and tyrosine phosphorylation sites in the N-terminal extension of GS do not affect its binding to CRBN. 293T cells stably expressing ^{Flag}CRBN were transfected with empty vector or plasmids encoding the indicated GS mutants for 48 h. Cell extracts were immunoprecipitated with anti-Flag antibody and the precipitated and input fractions were evaluated by SDS-PAGE and Western blot analysis with antibodies to the Myc and Flag tags. TS2-7A combines T2A, T3A, S4A, S6A, and S7A. TS2-19 combines TS2-7A plus S19A. TSYA combines TS2-19 plus Y17A.

(B) ^{HA}Ub analysis for Figure 4C. Western blot analysis showing ubiquitylation of input and precipitated proteins for the experiment shown in Figure 4C.

(C) p300 and CBP interact with GS. 293T cells were transfected with the indicated Myc-tagged GS and HA-tagged p300 or CPB plasmids. Immunoprecipitation was performed with anti-HA and the precipitated and input fractions were analyzed by SDS-PAGE and immunoblotting with anti-Myc and anti-HA antibodies. S.E., short exposure. L.E., long exposure.

(D) GS is acetylated *in vitro* by p300. *In vitro* acetylation assay using purified recombinant human GS tagged with 6 histidine residues (rGS^{6xHis}) was performed in the absence or presence of recombinant HAT domain of p300, followed by Western blot analysis with antibodies against acetylated lysine (Ac-Lys) and GS.

(E) Lysines 11 and/or 14 are acetylated. Lysates from CRBN-KO 293FT cells transfected with plasmids expressing Myc-tagged wild type GS or its AA mutant (K11A, K14A GS) were immunoprecipitated with anti-Myc beads, eluted with Myc peptide, and then analyzed by SDS-PAGE and immunoblotting with the indicated antibodies.

(F) Glutamine induces acetylation of endogenous GS. CRBN-KO 293FT cells were starved of glutamine for 24 h, then pre-treated with or without 10 μM p300/CBP inhibitor C646 in fetal bovine serum-free DMEM medium for 1 h, followed by additional treatment with 4 mM glutamine for 2 h. The cell lysates were subjected to immunoprecipitation (IP) by normal IgG or GS antibody, followed by SDS-PAGE and immunoblotting of precipitated and input fractions with the indicated antibodies.

(G) Purification of recombinant human GS**.** Fractions from purification of GS were analyzed by SDS-PAGE and staining with Coomassie Blue. This recombinant human GS was used in the *in vitro* acetylation assay, shown in Figure S4D.

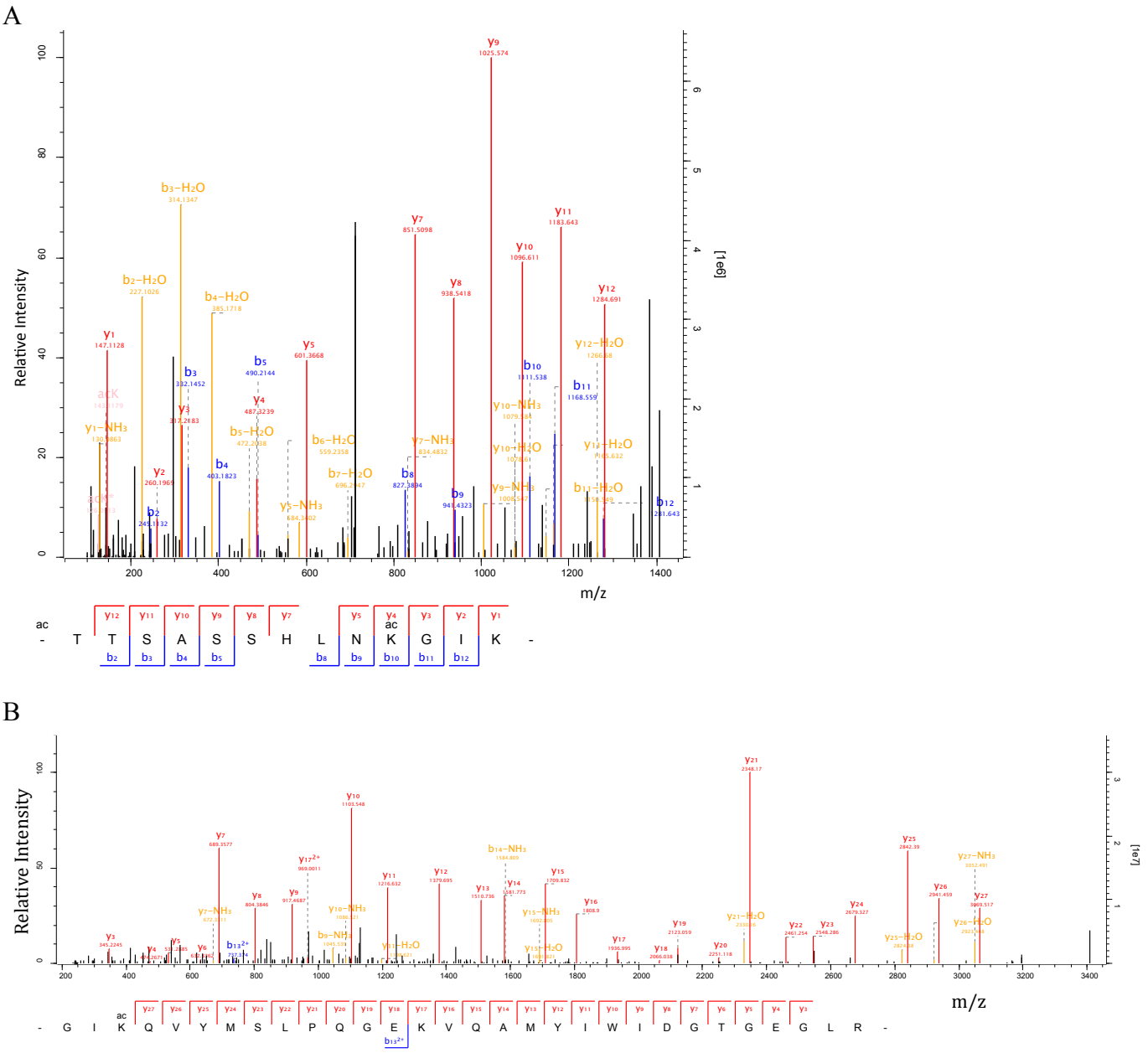


Figure S5. Identification of GS acetylation sites by mass spectrometry, related to Figure 5. Mass spectra of GS peptides containing acetylated K11 (A) or K14 (B). Samples were prepared from CRBN-KO 293FT cells transfected with GS^{Flag} and treated with HDAC inhibitors (1 μM TSA plus 10 mM NAM) for 12 h. As noted in the Results, we also obtained evidence for acetylation at K25, K189, K241, K268, and K291. However, we were unable to detect a peptide doubly acetylated at K11 and K14 even though it should, in theory, be detectable. We do not understand the reason for this but we note that singly acetylated peptides were detected at stoichiometries of ~0.2%, and so if the frequency of double acetylation is the product of the individual frequencies, the doubly acetylated species may be below our limit of detection. Alternatively, it is possible that acetylation at both residues is coupled to other covalent

modification(s) that generate a peptide of *m/z* that is not accounted for by our search algorithm. We did detect a peptide containing T2 at the N-terminus, that was doubly acetylated on T2 and K11. The strongest evidence in support of simultaneous acetylation at K11 and K14 being critical is that chemically-synthesized N-terminal peptides bound CRBN tightly only when both residues were acetylated.

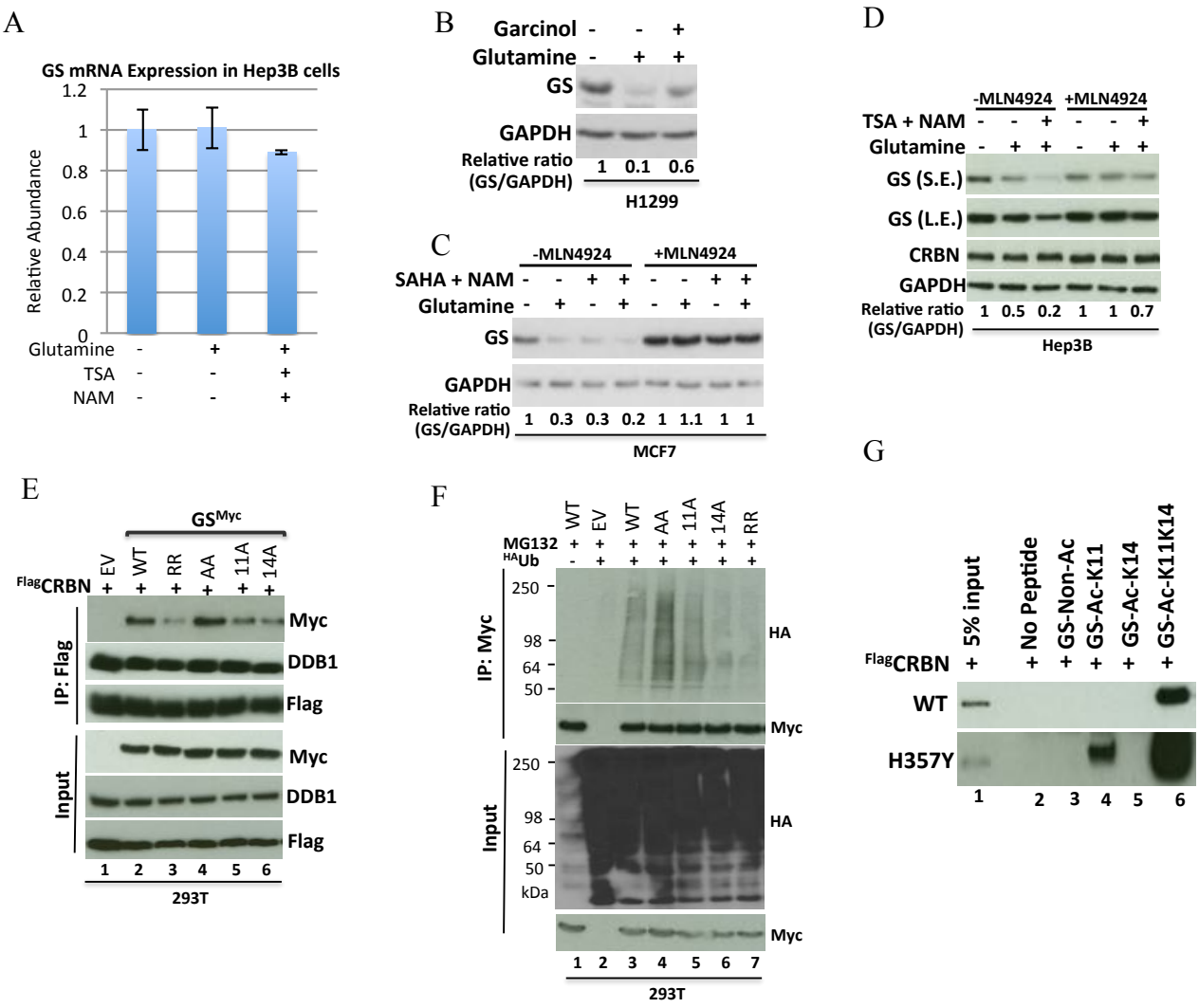


Figure S6. GS residues K11 and K14 each contribute to binding CRBN and subsequent ubiquitylation, related to Figure 5 and Figure 6.

(A) HDAC inhibitors do not act via reduction of *GS* mRNA transcript. Hep3B cells were starved of glutamine for 48 h, followed by incubation for 7 h with or without 4 mM glutamine in the presence or absence of HDAC inhibitors (1 μM TSA plus 10 mM NAM). *GS* mRNA expression was measured by quantitative RT- PCR. Gene expression was normalized to *GAPDH* mRNA level. The relative abundance is displayed as an average of triplicates. Error bars indicate ± SD. The results shown are representative of two independent experiments.

(B) The histone acetyltransferase inhibitor garcinol partially blocks glutamine-induced GS degradation. H1299 cells were starved of glutamine for 24 h, and then pretreated with or without garcinol (20 μM) for 1 h, followed by 4 mM glutamine treatment for 8 h. Cell lysates were analyzed by Western blots (WB) with antibodies against GS and GAPDH. The GS:GAPDH ratios, normalized to untreated cells, are shown below the bottom panel.

(C) MLN4924 inhibits glutamine- or HDACi-induced GS degradation. MCF7 cells were starved of glutamine for 24 h, and then pretreated with or without MLN4924 (2 μM) for 30 min, followed by HDAC inhibitor (2 μM SAHA and 10 mM NAM) and 4 mM glutamine treatment for 6 h. Cell lysates were analyzed by WB with antibodies against GS, CRBN, and GAPDH. The GS:GAPDH ratios, normalized to untreated cells, are shown below the bottom panel.

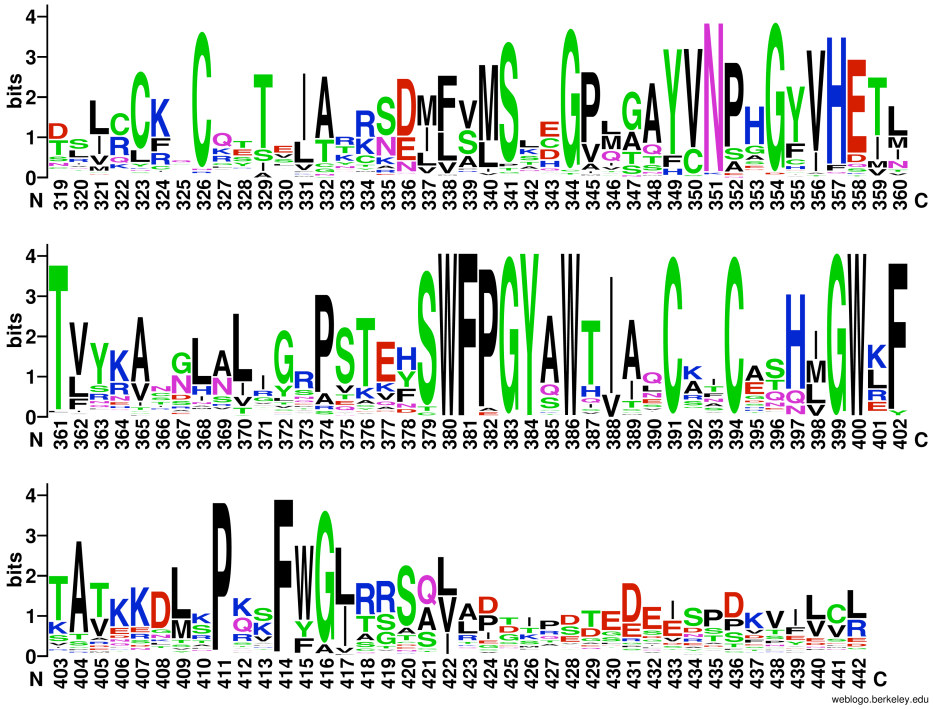
(D) MLN4924 diminishes HDAC inhibitor-induced GS degradation. Hep3B cells were starved of glutamine for 48 h. Starved cells were either mock treated or supplemented with HDAC inhibitors (1 μM TSA and 10 mM NAM) in the presence or absence of 2 μM MLN4924, as indicated, for 1 h, followed by addition of 4 mM glutamine for 7 h. Equal amounts of cell extracts were analyzed by WB with antibodies against GS and GAPDH. The GS:GAPDH ratios, normalized to untreated cells, are shown below the bottom panel. S.E., short exposure. L.E., long exposure.

(E) K11 and K14 individually contribute to CRBN binding. 293T cells stably expressing ^{Flag}CRBN were transfected with empty vector or plasmids encoding the indicated GS mutants for 48 h. Cell extracts were immunoprecipitated with anti-Flag antibody and the precipitated and input fractions were analyzed by WB with antibodies against DDB1 and the Myc and Flag tags. WT: wild type. RR: K11R, K14R GS. AA: K11A, K14A GS.

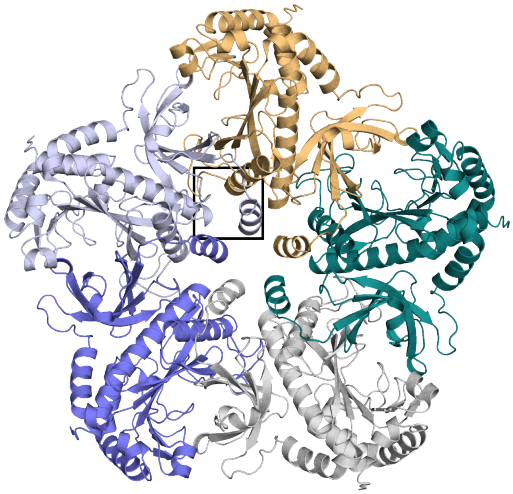
(F) K11 and K14 individually contribute to ubiquitylation. 293T cells were transfected with plasmids encoding ^{HA}Ub and the indicated Myc-tagged GS mutants. After 24 h of transfection, the cells were treated with 10 μM MG132 for 4 h, followed by cell lysis, denaturation of the lysate proteins, and IP with anti-Myc antibody. The input lysates and bound fractions were evaluated by SDS-PAGE and immunoblotting with antibodies against the HA and Myc tags.

(G) CRBN-H357Y can bind GS N-terminal peptide monoacetylated on K11. Pull-down assays were carried out using purified recombinant human ^{Flag}CRBN and immobilized GS peptides that are not acetylated, acetylated on K11 or K14, or acetylated on both residues, as indicated. Bound proteins were analyzed by WB with anti-Flag.

A



B



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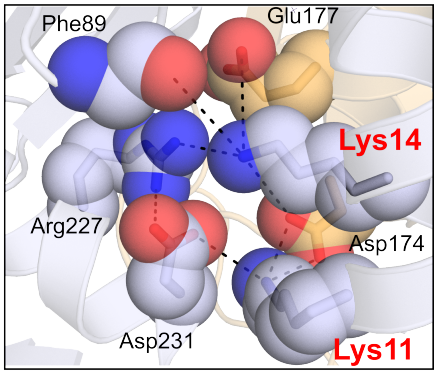

Figure S7. Conservation of the tri-Trp pocket in CRBN and structure of human GS, related to Figure 6 and Figure 7.

(A) The C-terminal domains of CRBN orthologs across animal and plant kingdoms show 100% conservation in the pocket formed by tryptophan residues W380, W386 and W400 (Sequence 319-360 top panel, sequence 361-402 middle panel, sequence 403-442 bottom panel). This is a

graphic representation of amino acid sequence alignment, generated using WebLogo (http://weblogo.berkeley.edu/logo.cgi).

(B-C) K11 and K14 participate in a network of electrostatic interactions at the interface between adjacent protomers of GS. (B) A GS pentamer is shown with each protomer in a different color (PDB#: 2QC8). (C) A network of electrostatic interactions coordinates K11 and K14. Silver and yellow indicate two neighboring GS subunits. K11 participates in an inter-molecular interaction with D174 of an adjacent protomer and an intra-molecular interaction with D231. K14 is buried within a hydrophilic pocket at the pentamer interface formed by the intra-subunit residue sidechains R227 and D231, the backbone of F89, and inter-subunit residue sidechains D174 and E177. Figure was made using MacPyMol: PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC.

Table S1, related to Figure 1. Changes in the level of CRBN interaction upon thalidomide treatment. Ratio (fold) represents mean ratio of the binding affinity of the protein to CRBN based on the presense/absense (+/-) of thalidomide (Thal).

Gene names	+/-Thal Ratio
GLNS (glutamine synthetase)	2.508063449
TCP1	0.892651756
CCT5	0.858384639
PDIA6	0.851535402
CCT6A	0.848325712
CCT8	0.836829057
RPS8	0.818644799
IMPDH2	0.813783598
RPSA;RPSAP58;LAMR1P15	0.800547102
RPL27	0.798727238
GLUD1;GLUD2	0.793470294
HSP90AB1	0.789986397
VCP;DKFZp434K0126	0.77019844
RPS3A	0.76069131
LUC7L2	0.757764973
ATP5A1	0.754135965
CMBL	0.746345707
HSP90AA1	0.745441526
STK38	0.744495026
RPS25	0.73481289
PRPS1	0.726353702
HNRNPU;HNRPU	0.713744524
TRIM21	0.712479811
C22orf28	0.710856769
RUVBL1	0.709889429
TXN	0.703663751
C11orf84	0.703209501
RPL26;KRBA2;RPL26L1	0.697997709
HNRNPM;ORF;HNRPM	0.697658496
RUVBL2	0.692702735
RPL35;LOC154880	0.683910758
KIF11	0.673725446
C12orf23	0.671822923
SPIN1	0.668859796
SNRPN;SNRPB	0.66049843
SNRPD3	0.658296855
SRSF3;SFRS3	0.650957681

PRPS2	0.644522087
DDX5;DKFZp686J01190	0.632144562
BAG2	0.6302998
CLNS1A	0.626981496
ATP5B	0.623583663
RIOK1	0.618678291
RBM39;DKFZp781C0423;DKFZp686A11192;DKFZp781I1140;DKFZp686C17209	0.618210651
SNRPD1	0.616791668
IVNS1ABP	0.613076831
U2AF2	0.612315329
SNRPF	0.60714932
BOLA2	0.598857168
SF3A3	0.595900095
HNRNPK;HNRPK	0.590544635
WDR77	0.589527623
FUS	0.580689474
SFRS11;SRSF11	0.572955238
HNRNPAB	0.569043974
COMT	0.548219847
PUF60	0.547071669
DKFZp686K23100;MATR3;DKFZp686K0542	0.525988372
PIH1D1	0.515270413
RBMX;RBMXL1	0.500368967
HNRNPA2B1	0.453275527
HNRNPL	0.439962795
HNRNPC;hCG_1641229;HNRPCL1;HNRNPCL1;LOC440563;LOC649330	0.422945565

Table S2, related to Figure 2. GS is highly transcribed in brain, liver, kidney, lung and skeletal muscle. GS mRNA expression profile in diverse murine tissues reported at BioGPS.org.

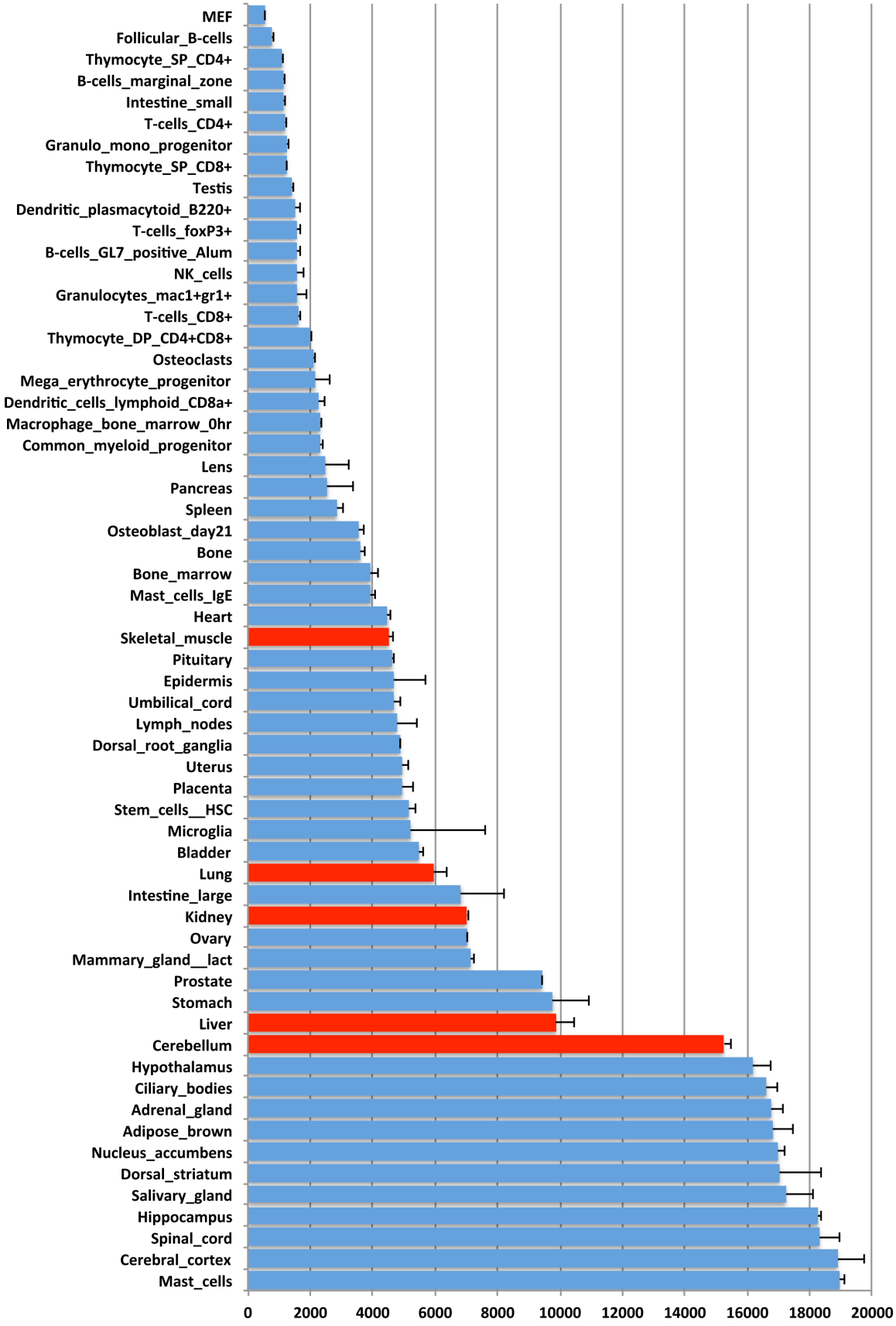

Table S3, related to Figure 2. Quantification of glutamine and glutamate in serum of wild-type (WT) and homozygous mutant CRBN$^{-/-}$ (HM) mice (concentration original serum sample).

sample ID	mean ± SD (ng/mL, *n*=3)		Q/E ratio	sample ID	mean ± SD (ng/mL, *n*=3)		Q/E ratio
	Q	E			Q	E	
WT 1	97.6 ± 1.5	8.9 ± 0.5	11.0	HM 1	107.6 ± 2.4	6.6 ± 0.2	16.2
WT 2	88.0 ± 2.0	6.2 ± 0.4	14.3	HM 2	92.0 ± 6.4	5.1 ± 0.5	18.1
WT 3	96.4 ± 0.2	6.7 ± 0.2	14.5	HM 3	112.6 ± 0.7	6.0 ± 0.1	18.9
WT 4	93.1 ± 0.6	8.1 ± 0.1	11.5	HM 4	92.2 ± 0.5	5.7 ± 0.1	16.2
WT 5	83.3 ± 0.8	7.2 ± 0.1	11.7	HM 5	96.0 ± 0.9	6.4 ± 0.2	15.1
WT 6	72.1 ± 0.7	5.0 ± 0.2	14.4	HM 6	117.0 ± 1.3	9.8 ± 0.2	11.9

Table S4, related to Figure 5. Identification of lysine acetylation sites on GS^{Flag} isolated from CRBN-KO 293FT cells by mass spectrometry. The value for the posterior error probability (PEP) is shown.

Positions of lysine residues	PEP Value
11	2.78E-44
14	2.08E-28
25	1.53E-42
189	5.25E-42
241	1.16E-05
268	0.000177049
291	1.06E-12

Table S5. List of plasmids used in this study with their corresponding Deshaies laboratory database (RDB) number.

RDB #	Plasmid Name
2910	pCMV6-CRBN-Myc-Flag
2911	pCMV6-GS-Myc-Flag
2915	pCDH-Flag-CRBN
2916	pCDH-FlagCRBN$^{YW/AA}$ mut
2918	CRBN_1 shRNA: V2LHS_226831
2919	CRBN_5 shRNA: V3LHS_395310
3208	CRBN_2 shRNA: V2LHS_115329
3209	CRBN_3 shRNA: V2LHS_224589
3210	CRBN_4 shRNA: V2LHS_226831
3167	pCMV6-GS-Myc
3168	pCMV6-GS Myc-K11R/K14 R mutant (RR)
3169	pCMV6-GS Myc-K11A/K14 A mutant (AA)
3170	pCMV6-GS Myc-K11A mutant (K11A)
3171	pCMV6-GS Myc-K14A mutant (K14A)
3172	pCMV6-GS Myc-d1
3173	pCMV6-GS Myc-d2
3174	pCMV6-GS Myc-d3
3175	pCMV6-GS Myc-d4
3176	pCDH-^{FH}GS
3177	pCDH- d1^{FH}GS
3178	pCDH-^{Myc}GFP
3179	pCDH- GS-N^{WT}-^{Myc}GFP
3180	pCDH- GS-N^{RR}-^{Myc}GFP
3181	pCDH-Flag-CRBN- Δ1
3182	pCDH-Flag-CRBN- Δ2
3183	pCDH-Flag-CRBN- Δ3
3184	pCDH-Flag-CRBN- Δ4
3185	pCDH-Flag-CRBN-W386E
3186	pCDH-Flag-CRBN-W400E
3187	pCDH-Flag-CRBN-N351R
3188	pCDH-Flag-CRBN-H357Y
3189	pVL1393-CUL4A
3190	pVL1393-GS-6xHis
3191	pcDNA3-p300-HA
3192	pcDNA3-CBP-HA
3193	pCMV6-GS-Myc-Flag K11R/K14R mutant

Table S6. Mammalian Cell lines used in this study with their corresponding Deshaies laboratory database (DTC) number.

DTC#	Cell Line Name
147	HEK293T cells expressing ^{Flag}CRBN Wild-type
148	CRBN-KDO 293T
150	MM.1S
151	CRBN-KDO MM.1S
227	Hep3B
228	shRNA Control Hep3B
229	*CRBN*shRNA_4 Hep3B
217	*CRBN*shRNA_5 Hep3B
164	CRBN-KO 293FT
231	CRBN-knockout Hep3B
232	*CRBN*shRNA_5 NCI-H1299
47	NCI-H1299
72	A549
43	ZR-75-1
63	MCF7
64	MDA-MB-231
68	T47D
78	SKBr
50	A172
76	U87

Supplemental Experimental Procedures

Materials and cell lines

Thalidomide (Tocris Cookson), lenalidomide (Chem-Pacific), pomalidomide (Selleck Chemicals), Trichostatin A and Suberoylanilide hydroxamic acid (SAHA; Vorinostat) from Sigma, C646 (a competitive histone acetyltransferase (HAT) p300/CBP inhibitor, Sigma), garcinol (a p300 and PCAF histone acetyltransferase inhibitor; Santa Cruz Biotechnology), MLN4924 (Pevonedistat) from Active Biochem, MG132 (Millipore), and Bortezomib (LC Laboratories) were dissolved in dimethyl sulfoxide (DMSO) at room temperature and were stored at −80°C until use. Nicotinamide and Cycloheximide (Sigma) were dissolved in distilled water and kept at 4°C and -20^{0}C, respectively.

MM.1S, a human multiple myeloma (MM) cell line was purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). Cells were maintained in RPMI-I640 medium containing 10% (v/v) heat-inactivated fetal bovine serum (Gibco, Grand Island, NY, USA) supplemented with 2 mM glutamine and penicillin-streptomycin. Hep3B cells (ATCC # HB-8064), NCI-H1299 cells (ATCC# CRL-5803), A549 cells (ATCC# CCL185), ZR-75-1 cells (ATCC# CRL-1500), MCF7 cells (ATCC# HTB-22), MDA-MB-231 cells (ATCC# HTB-26), T47D cells (ATCC# HTB133), SKBr cells (ATCC# HTB30), A172 cells (ATCC# CRL-1620) and U87 cells (ATCC# HTB-14) were purchased from ATCC (American Type Culture Collection, Manassas, VA, USA). *CRBN*-knockout 293FT cells were kindly provided by Prof. William Kaelin. HEK–293T cells were purchased from ATCC. Cells were grown in DMEM supplemented with 10% FBS, 2 mM glutamine and penicillin-streptomycin. All cell lines were checked periodically to ensure they were free of mycoplasma contamination. Cell lines were also authenticated by short tandem repeat (STR) DNA profiling analysis by Laragen, Inc.

Antibodies

Anti–Flag (M2, F3165) was from Sigma. Anti–HA (influenza hemagglutinin) (16B12, MMS-101P) was from Covance. Anti-Myc (9E10) was from Santa Cruz Biotechnology. Anti-DDB1 (ab21080) was from Abcam. Anti-glutamine synthetase (C-20; sc-6640-R) used for Western blot was from Santa Cruz Biotechnology. Anti-glutamine synthetase (8G9; LF-MA0095) used for immunoprecipitation (IP) was from Thermo Fisher Scientific. Mouse monoclonal anti-CRBN antibody against amino acids 1-18 of human CRBN was described previously (Lopez-Girona et al., 2012; Zhu et al., 2011). Anti-CRBN antibody (HPA045910) was from Sigma. Anti-GAPDH (MAB374) was from Millipore. Anti-acetylated-lysine antibody (9441s) was from Cell Signaling.

Plasmids

Human CRBN and GS expression vectors pCMV6-CRBN-Myc-Flag and pCMV6-GS-Myc-Flag (C-terminal Myc- and Flag-tagged) were purchased from OriGene. pCMV6-GS-Myc was generated by introducing a STOP codon between Myc and Flag by using a QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). All GS deletions or single-point mutations in pCMV6-GS-Myc vectors were generated using Q5 Site-Directed Mutagenesis Kit (New England BioLabs). Lentiviral vectors directing expression of wild-type ^{Flag}CRBN and its mutants were constructed in pCDH-T2AcGFP-MSCV (System Biosciences). Chimeric fusion proteins, consisting of the N-terminal region of GS (amino acids 1-25) wild type (GS-N^{WT}) or RR mutant (GS-N^{RR}) fused to Myc-tagged GFP, were constructed in pCDH-T2AcGFP-MSCV. All cDNAs cloned into mammalian expression vectors and lentiviral expression vectors were confirmed by DNA sequencing.

Generation of stable cell lines expressing ^{Flag}CRBN and its mutants

The lentiviruses were generated according to the manufacturer's protocol (System Biosciences). Briefly, lentiviral plasmids were co-transfected with psPAX2 (packaging plasmid) and pMD.2G (enveloping plasmid) into HEK293T cells using Fugene HD (Promega). Virus-containing supernatants were harvested at 48 h and 72 h post transfection. The lentiviruses were precipitated using PEG-it Virus Precipitation Solution according to the manufacturer's protocol (System Biosciences). Infection efficiency was >95% as judged by fluorescence microscopy and CRBN expression was confirmed by immunoblot.

Identification of ^{Flag}CRBN binding partners by mass spectrometry

SILAC-labeled cultures of the stable cell lines were grown as described previously (Lee et al., 2011). Briefly, HEK293T cells, stably expressing ^{Flag}CRBN, were cultured in medium formulated with isotopically light lysine and arginine ('light' medium) or in medium formulated with isotopically heavy lysine and arginine ('heavy' medium). The cells grown in 'heavy' medium were treated with thalidomide (a final concentration of 50 μM) for 4 hours, while the cells growing in 'light' medium were treated with DMSO. SILAC experiments were repeated after swapping the SILAC labels in which the cells cultured in 'light' and 'heavy' medium were treated with thalidomide and DMSO, respectively. The ^{Flag}CRBN immunoprecipitates were prepared, mixed, digested with trypsin, and analyzed by quantitative mass spectrometry (Lee et al., 2011).

Thermo raw files were processed and searched with MaxQuant (v. 1.4.1.2) (Cox and Mann, 2008; Cox et al., 2011). Trypsin was specified as the digestion enzyme with up to two missed cleavages. Protein N-terminal acetylation (+42.0106) and methionine oxidation (+15.9949) were

specified as variable modifications. Carbamidomethylation of cysteine (+57.0215) was specified as a fixed modification. Arg6 (+6.0138) and Lys8 (+8.0142) were specified as the SILAC labels. Requantification and match between runs were enabled. Precursor ion tolerance was 7ppm and fragment ion tolerance was 0.5 Da. All human Uniprot (Apweiler et al., 2013) entries were searched (148298 sequences, downloaded on 05Dec12) along with a contaminant database containing proteins such as keratin and trypsin (247 sequences). Additionally, to determine the false discovery rate, a decoy database was constructed by reversing the target database. While no minimum score was specified, the protein and peptide level false discovery rates were fixed at 1% and we required that all proteins reported were identified in both biological replicates by at least two peptides.

Proteins were quantified by first calculating the median of all peptide ratios within each biological replicate and then calculating the mean of the two biological replicate ratios. Only peptides uniquely assignable to the protein group were used for quantification. Ratios were normalized in each biological replicate so that the bait (CRBN) had a ratio of 1. Individual ratio measurement error was estimated using pooled variance and overall ratio standard error was calculated using bootstrap analysis. P-values were calculated using a z-test where the null hypothesis was the protein was unchanged (i.e., had a ratio of 1). Q-values were calculated from the p-values using the Storey method for calculating false discovery rates (Storey, 2002). A q-value of 0.05 was used as a threshold for significance.

Identification of acetylated lysines on GS by mass spectrometry

Chemicals and Reagents—Sequencing-grade trypsin (V5117) and mass spec-grade Lysyl endopeptidase (V1671) were purchased from Promega. Mass spec-grade water (39253), mass

spec-grade acetonitrile (34967), and *n*-dodecyl-β-D-maltoside (89903) were obtained from Thermo Scientific.

Immunoprecipitation— *CRBN*-KO 293FT cells were transiently transfected with a plasmid expressing GS^{Flag}. After 36 hours of transfection, cells were treated with HDAC inhibitors (1 μM TSA plus 10 mM NAM) for 12 hours. The cell pellets were lysed in lysis buffer (50 mM HEPES, pH 7.5, 70 mM KOAc, 5 mM $Mg(OAc)_2$, 0.2% *n*-dodecyl-β-D-maltoside) containing protease inhibitor (Roche; 04693159001) and HDAC inhibitors (1 μM TSA plus 10 mM NAM) for 30 min on a gyrating platform at 4 °C. The lysates were centrifuged at 16,600 x *g* for 15 min to remove cell debris, and the supernatant was incubated with anti-FLAG M2 affinity gel (Sigma; A2220) on a gyrating platform for 2 hr at 4 °C. The anti-FLAG M2 affinity gel was washed with 20 bed volumes of the lysis buffer three times and then with 100 mM Tris–HCl (pH 8.5) two times, followed by a 15-min elution with 100 mM Tris–HCl (pH 8.5) containing 10 M urea.

Mass Spectrometric Analysis— Eluted proteins were digested at 37 °C first with Lys-C (200 ng) for 4 hr in the elution buffer with 8 M urea, and then with trypsin (200 ng) in the same buffer at 37°C overnight after diluting the urea concentration to 2 M. Following digestion, the tryptic peptides were desalted using Stagetips packed with reversed-phase C18 material (3M; 2215-C18) and concentrated using a vacuum concentrator. Dried samples were acidified by 0.1% formic acid prior to mass spectrometric analysis. All liquid chromatography-mass spectrometry experiments were performed using an EASY-nLC 1000 (Thermo Scientific) coupled to Q-Exactive mass spectrometer (Thermo Scientific) equipped with a home-made nano-electrospray ion source. Peptides were separated on a 15-cm reversed phase analytical column (75-μm internal diameter) packed in-house with C18 beads (3 μm, 100 Å; Agela technologies) using a

160-min gradient from 9.5% to 36.5% acetonitrile in 0.1% formic acid at a flow rate of 350 nL/min. The mass spectrometer was operated in data-dependent mode to automatically switch between full-scan MS and tandem MS acquisition. Survey full scan mass spectra were acquired in an Orbitrap (300–1800 *m/z*) using automated gain control target of 1,000,000 ions and a resolution of 70,000. The top twelve most intense ions from the survey scan were isolated with automated gain control target of 500,000 ions and a resolution of 35,000. The isolated ions were fragmented in the high collision dissociation cell by collisionally-induced dissociation with 27% normalized collisional energy and 2 *m/z* isolation width. Precursor ion charge state screening was enabled and all singly-charged and unassigned charge states were rejected. The dynamic exclusion list was set with a maximum retention time of 30 sec.

Data Analysis—The resulting raw data files were searched using MaxQuant (version 1.5.3.8) (Cox and Mann, 2008) against the IPI human database (v3.87). The search parameters were Lys-C and tryptic digestion, maximum of two missed cleavages, fixed carboxyamidomethyl modifications of cysteine, variable oxidation of methionine, variable acetylation of lysine, variable carbamylation of lysine, variable carbamylation of protein N-termini, and variable acetylation of protein N-termini. Mass tolerances for precursor ions were 4.5 ppm and those for fragment ions were 20 ppm.

CRBN knockdown

The lentiviral constructs expressing nontargeting (control, CT) and human *CRBN* shRNAs (CRBN_1 shRNA: V2LHS_226831; CRBN_2 shRNA: V2LHS_115329; CRBN_3; shRNA: V2LHS_224589; CRBN_4 shRNA: V3LHS_413798; CRBN_5 shRNA: V3LHS_395310) in the pGIPZ lentiviral vector were purchased from Open Biosystems. Five lentiviruses targeting

CRBN were screened to identify shRNAs that optimally suppressed CRBN. Virus preparation and cell infection were performed according to the manufacturer's protocol, with minor modifications. Briefly, shRNA-encoding plasmids were co-transfected with psPAX2 (packaging plasmid) and pMD.2G (enveloping plasmid) into HEK293T cells using Fugene HD (Promega). Virus-containing supernatants were harvested at 48 h and 72 h post transfection. The lentiviruses were precipitated using PEG-it virus precipitation solution according to the manufacturer's protocol (System Biosciences), and target cells were infected in the presence of 8 μg/ml polybrene. After 24 hours of transduction, the cells were selected with puromycin (1 μg/ml for MM.1S cells, and 2-4 μg/ml for 293T cells, Hep3B cells and H1299 cells) for 2 weeks. Knockdown efficiencies were analyzed by immunoblot.

Generation of CRBN knockout Hep3B cells by CRISPR/Cas9

Two pairs of single-guide RNAs (sgRNAs) (CRBN sgRNA_1 Forward 5'- CAC CGG TGT CAA AAT TTA TGA TGT T -3', CRBN sgRNA_1 Reverse 5'- AAA CAA CAT CAT AAA TTT TGA CAC C -3'; CRBN sgRNA_2 Forward 5'- CAC CGA ACC ACC TGC CGC TCC TGC C -3', CRBN sgRNA_2 Reverse 5'- AAA CGG CAG GAG CGG CAG GTG GTT C -3') were designed for targeting the human CRBN genomic locus as previously described (Shalem et al., 2014). The annealed oligonucleotide pairs were cloned into a lentiCRISPR vector expressing Cas9 (Shalem et al., 2014). Lentiviruses were generated by using a similar protocol described above. Infected Hep3B cells were selected with puromycin (3 μg/ml) for 2-3 weeks and tested for CRBN expression by immunoblot analysis.

Protein expression and purification of recombinant proteins

Human *RBX1* cDNA was cloned into pAcG2T baculovirus transfer vector (BD Bioscience) with an N-terminal GST tag followed by a TEV cleavage site. Human *CUL4A* or *GLUL*-6xHis cDNA was cloned into pVL1393 baculovirus transfer vector (BD Bioscience). Baculoviral stocks were generated by co-transfecting the transfer vector with the linearized ProGreen helper vector (AB Vector). Large-scale viral stocks were created following two rounds of viral amplification. Proteins were expressed in Hi-Five cells (Invitrogen). CUL4A-RBX1 was purified on glutathione resin followed by digestion with TEV protease and size exclusion chromatography (Superdex 200; GE Healthcare). GS-6xHis was purified on Ni-NTA affinity resin, followed by size exclusion chromatography (Superdex 200; GE Healthcare).

Immunoblot analysis

Cells were lysed in RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 1% Sodium deoxycholate, 0.1 % SDS, [pH 7.5]) supplemented with complete protease inhibitor cocktail (Roche). Lysates were clarified by centrifugation at 13,000 RPM for 10 min. Proteins were quantified by the Bradford method (Bio-Rad, Hercules, CA). Equal amounts of protein (10-40 μg/lane) were separated by SDS-PAGE and transferred to a PVDF membrane. The membranes were blotted with the indicated antibodies. Anti-rabbit or anti-mouse antibodies conjugated to horseradish peroxidase (Vector Labs) were used as secondary antibodies, and the signal was detected using a Super Signal West Pico Substrate kit (Fisher Scientific).

Immunoprecipitation

Cells were lysed in immunoprecipitation (IP) buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 0.5% NP-40) containing a protease inhibitor cocktail, followed by centrifugation at 13,000 RPM for 10

min. Proteins were immunoprecipitated with the indicated antibodies for 2-4 hours at 4^0C, followed by incubation with protein G sepharose 4 Fast Flow (GE Healthcare) for 1 hour at 4^0C. After binding, the beads were washed 3x in IP buffer containing 300 mM NaCl (stringent washing). Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting with the indicated antibodies.

Experimental animals

The generation and screening of *CRBN*-knockout (KO) mice were described previously (Lee et al., 2013). Wild-type and *CRBN*-KO mice (background of C57BL/6) were maintained on a standard chow diet and water *ad libitum* in pathogen-free conditions with a 12-hour light-dark cycle. All experiments were approved by the Gwangju Institute of Science and Technology Animal Care and Use Committee. To detect endogenous GS protein levels, male mice (8 weeks old; n=3-4 per group) were fasted for 24 hours and fed 4 hours for refeeding. Protein extracts were prepared from various mouse tissues and quantified by the Bradford method (Bio-Rad Laboratories). An equal amount of proteins was separated by SDS-PAGE and subjected to Western immunoblotting.

Quantification of glutamine (Q) and glutamate (E) in mouse serum by LC-MS/MS

Q and E in serum of wild-type (WT) and homozygous *CRBN*-KO (HM) mice were determined using isotope dilution (ID) LC-MS/MS. For the accurate and precise quantification of Q and E, isotope labeled Q and E (Q*, E*) were used as internal standards. The isotope dilution (ID) method is the most effective way to reduce uncertainties throughout the whole experimental

procedure since the isotopic analogues exhibit the same chemical properties as those of unlabeled compounds, except for molecular mass (Burkitt et al., 2008; Jeong et al., 2011).

All of standard solutions and internal standard solution were prepared gravimetrically, and concentrations were adjusted to Q and E in the working serums. A working internal standard solution was prepared gravimetrically from isotope labeled L-glutamine ($^{13}C_5$, 99%; Q*) and L-glutamate ($^{13}C_5$, 99% and $^{15}N_1$, 99%; E*) purchased from Cambridge Isotopes Laboratory (Andover, MA, USA), and concentrations were adjusted to Q and E in the working serum. Standard solutions for calibration curves were also prepared in the manner of gravimetry with L-glutamine (Q) and L-glutamate (E) obtained from Sigma Aldrich (St. Louis, MO, USA). Serum was diluted tenfold with water as a working sample. Twenty-five microliters each of diluted serum or standard solution and internal standard solution were gravimetrically added to each tube and mixed well. Acetonitrile (200 μL) was added to yield a final concentration of 80% v/v. The tubes were sealed and mixed again followed by centrifugation for 20 min at 13,000 rpm. The supernatant was evaporated and reconstituted with 200 μL water. The samples were filtered through a disposable sample filter, and then injected into the LC-MS/MS system.

Ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) analysis was performed using an ACQUITY series UPLC system coupled in-line to a Xevo TQ-S MS system (Waters, Massachusetts, USA). Chromatographic separation was performed using a KINETEX C18 column (150 mm × 2.1 mm I.D., 2.6 μm particle size; Phenomenex, Torrance, CA, USA) connected to a guard column unit (Security Ultra, Phenomenex). Isocratic elution with the mobile phase comprised 0.3% (v/v) heptafluorobutyric acid in 10 mM ammonium formate was adopted for separation with a flow-rate of 400 μL/min. The total run time was 3 min per injection, the sample injection volume was 1 μL, and the column oven temperature was

maintained at 40°C. MS measurements of the AAs were performed on a triple-quadrupole mass spectrometer equipped with a turbospray source in multiple reaction monitoring (MRM) mode. The precursor to product ion transitions that were monitored were as follows: Q; 147.1 > 84.1, Q*; 152.1 > 88.0, E; 148.0 > 84.1, E*; 154.1 > 89.0. Source ionization and fragmentation parameters were finely tuned by monitoring the MS signal prior to sample analysis. Peaks of unlabeled and isotope-labeled compounds were integrated and the ratio of these two areas was calculated. The peak area ratios were calculated against the calibration curves derived from theoretical concentration and peak area ratio of the standard solutions.

Cycloheximide chase experiments

Cells were seeded overnight in complete medium in 24-well plates (1×10^5 cells/well), and then treated with 100 μg/ml cycloheximide (CHX). At the indicated times following addition of CHX, samples were harvested for immunoblot analysis.

***In vivo* ubiquitylation assay.**

HEK293T stable cell lines expressing nontarget shRNA (Control, CT) or *CRBN* shRNAs (a combination of *CRBN*_1 plus *CRBN*_5) were transiently transfected in 10-cm plates with plasmids that expressed ^{Flag}GS (6 μg) and ^{HA}ubiquitin (3 μg). After 30 hrs of transfection, the cells were treated with DMSO or MG132 (10 μM) for 3 hours. Then, the cells were lysed in 0.3 ml denaturing IP lysis buffer (1% SDS, 50 mM Tris, 10 mM DTT, [pH 7.5]) and boiled for 5 minutes. Subsequently, denatured proteins were diluted 10x in immunoprecipitation buffer and immunoprecipitated with anti-Flag resin. IP washing steps were performed using IP lysis buffer

supplemented with 0.5 M NaCl. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting.

***In vivo* acetylation assay**

CRBN-KO 293FT cells were transiently transfected with indicated plasmids. After 48-hour transfection, cells were lysed in RIPA buffer containing a protease inhibitor cocktail, 10 mM nicotinamide and 500 nM trichostatin A, and immunoprecipitated with the indicated antibodies. Immunoprecipitated proteins were resolved by SDS-PAGE, transferred to a PVDF membrane, and analyzed by immunoblotting.

***In vitro* acetylation assay**

The assays were performed as described previously (Van Nguyen et al., 2012). Briefly, 2 μg of human recombinant GS^{6xHis} purified from insect cells were mixed with 2 μg p300, HAT domain (Catalog #: 14-418, Millipore) in 50 μl acetylation assay buffer (50 mM Tris-Cl pH 8, 10% glycerol, 10 mM butyric acid, 0.1 mM EDTA, 1 mM DTT and protease inhibitor cocktail) supplemented with 100 μM acetyl CoA (Sigma). The mixture was then incubated for 60 min at 30°C on a rotating platform, followed by addition of 2x SDS-PAGE sample buffer (50 μl), separation of proteins by SDS-PAGE, and transfer to a PVDF membrane. Acetylation of GS^{6xHis} was detected by antibody specific for acetylated lysines.

RNA extraction and real time PCR assay.

Total RNA was extracted using the RNeasy Mini Kit from QIAGEN and converted into cDNA using Advantage RT-for-PCR Kit (Clontech) according to the protocols described in the

handbooks. Quantitative RT-PCR was performed using TaqMan gene expression assay (Applied Biosystems) and analyzed on the GeneAmp 7700 sequence detection system (Applied Biosystems). Gene expression was normalized to GAPDH mRNA level. The relative abundance is shown as an average of triplicates of quantitative PCR in each sample, and error bars indicate ± SD. Human GAPD (GAPDH) endogenous control (4326317E) and human glutamine synthetase (Hs00365928_g1) primers were purchased from Applied Biosystems.

Statistical analysis

Data are presented as mean ± one standard deviation (SD); p values were calculated using an unpaired two-tailed Student's t test in the Microsoft Excel software. $P > 0.05$ was considered to be not significant; $P < 0.05$ and $P < 0.01$ means significant and very significant, respectively.

Supplemental References

Apweiler, R., Martin, M.J., O'Donovan, C., Magrane, M., Alam-Faruque, Y., Alpi, E., Antunes, R., Arganiska, J., Casanova, E.B., Bely, B.*, et al.* (2013). Update on activities at the Universal Protein Resource (UniProt) in 2013. Nucleic Acids Research *41*, D43-D47.

Burkitt, W.I., Pritchard, C., Arsene, C., Henrion, A., Bunk, D., and O'Connor, G. (2008). Toward Systeme International d'Unite-traceable protein quantification: from amino acids to proteins. Anal Biochem *376*, 242-251.

Cox, J., and Mann, M. (2008). MaxQuant enables high peptide identification rates, individualized p.p.b.-range mass accuracies and proteome-wide protein quantification. Nat Biotechnol *26*, 1367-1372.

Cox, J., Neuhauser, N., Michalski, A., Scheltema, R.A., Olsen, J.V., and Mann, M. (2011). Andromeda: a peptide search engine integrated into the MaxQuant environment. J Proteome Res *10*, 1794-1805.

Jeong, J.S., Lim, H.M., Kim, S.K., Ku, H.K., Oh, K.H., and Park, S.R. (2011). Quantification of human growth hormone by amino acid composition analysis using isotope dilution liquid-chromatography tandem mass spectrometry. J Chromatogr A *1218*, 6596-6602.

Lee, J.E., Sweredoski, M.J., Graham, R.L., Kolawa, N.J., Smith, G.T., Hess, S., and Deshaies, R.J. (2011). The steady-state repertoire of human SCF ubiquitin ligase complexes does not require ongoing Nedd8 conjugation. Molecular & cellular proteomics : MCP *10*, M110 006460.

Lee, K.M., Yang, S.J., Kim, Y.D., Choi, Y.D., Nam, J.H., Choi, C.S., Choi, H.S., and Park, C.S. (2013). Disruption of the cereblon gene enhances hepatic AMPK activity and prevents high-fat diet-induced obesity and insulin resistance in mice. Diabetes *62*, 1855-1864.
Lopez-Girona, A., Mendy, D., Ito, T., Miller, K., Gandhi, A.K., Kang, J., Karasawa, S., Carmel, G., Jackson, P., Abbasian, M.*, et al.* (2012). Cereblon is a direct protein target for immunomodulatory and antiproliferative activities of lenalidomide and pomalidomide. Leukemia *26*, 2326-2335.
Shalem, O., Sanjana, N.E., Hartenian, E., Shi, X., Scott, D.A., Mikkelsen, T.S., Heckl, D., Ebert, B.L., Root, D.E., Doench, J.G.*, et al.* (2014). Genome-scale CRISPR-Cas9 knockout screening in human cells. Science *343*, 84-87.
Storey, J.D. (2002). A direct approach to false discovery rates. J Roy Stat Soc B *64*, 479-498.
Van Nguyen, T., Angkasekwinai, P., Dou, H., Lin, F.-M., Lu, L.-S., Cheng, J., Chin, Y.E., Dong, C., and Yeh, E.T. (2012). SUMO-specific protease 1 is critical for early lymphoid development through regulation of STAT5 activation. Molecular cell *45*, 210-221.
Zhu, Y.X., Braggio, E., Shi, C.X., Bruins, L.A., Schmidt, J.E., Van Wier, S., Chang, X.B., Bjorklund, C.C., Fonseca, R., Bergsagel, P.L.*, et al.* (2011). Cereblon expression is required for the antimyeloma activity of lenalidomide and pomalidomide. Blood *118*, 4771-4779.